The tumor promoter 12-O-tetradecanoylphorbol-13-acetate blocks differentiation of HT-29 human colon cancer cells

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SUMMARY

Recently developed HT-29-derived cell lines, which display variable differentiated phenotypes provide an invaluable opportunity to analyze the mechanism by which cell differentiation is regulated in the intestine. We have studied the effects of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) in the differentiation phenotype of mucus-secreting (HT-29 M6) and absorptive (HT-29 M3) cells. TPA prevented the accumulation of differentiation markers such as dipeptidylpeptidase IV, villin or mucins, down-regulated the expression of these molecules in post-confluent differentiated cell cultures and induced the loss of the functional integrity of the tight junction in the monolayer (i.e. decreased transepithelial resistance and inhibited dome formation). These effects were mediated by activation of protein kinase C (PK-C), as demonstrated using the specific inhibitor GF109203x. Analysis by immunoblotting of the PK-C isoforms present in HT-29 M6 cells revealed that the most abundant TPA-sensitive isoform was PK-C ε, although low levels of cPK-C were also detected. Further studies are necessary to elucidate the role of the different PK-C isoforms in the differentiation of HT-29 cells.

Key words: HT-29 cells, intestinal cells differentiation, TPA tumor promoter

INTRODUCTION

The intestinal crypt is a system perfectly regulated to maintain a precise balance between proliferation and differentiation (Potten and Loeffler, 1990). In this system, proliferation is restricted to the mid-crypt region; enterocytes, goblet cells and enteroendocrine cells differentiate as they migrate in vertical bands from the crypt to the extrusion zone at the tip of the villus, where they are subsequently exfoliated (Potten and Loeffler, 1990). The study of intestinal epithelial cell differentiation in vitro has been hampered by the lack of reproducible methods for the culture of normal intestinal epithelium and the undifferentiated phenotype of most colorectal cancer cell lines (Neutra and Louvard, 1991; Zweibaum et al., 1991). The study of intestinal epithelial cell differentiation in vitro has been hampered by the lack of reproducible methods for the culture of normal intestinal epithelium and the undifferentiated phenotype of most colorectal cancer cell lines (Neutra and Louvard, 1991; Zweibaum et al., 1991). Intestinal epithelial cell lines capable of differentiating in culture into goblet or absorptive cells have been recently described (Neutra and Louvard, 1991; Zweibaum et al., 1991). Several of them were isolated from the undifferentiated colon cancer cell line HT-29 when cultured under stress conditions (Neutra and Louvard, 1991; Zweibaum et al., 1991; Pinto et al., 1982; Augeron and Laboisse, 1984; Huet et al., 1987; Lesuffleur et al., 1990). Most cells in HT-29 cultures are undifferentiated and this cell line has been considered a model of multipotent proliferant mid-crypt-like cells (Zweibaum et al., 1991, Pinto et al., 1982; Augeron and Laboisse, 1984; Huet et al., 1987; Lesuffleur et al., 1990; Lesuffleur et al., 1991; Wice and Gordon, 1992). From a small percentage (less than 5%) of cells in the original HT-29 population, cultures with the ability to differentiate were isolated (Lesuffleur et al., 1991). After selection, the phenotype of these cell lines remains stable even when cultured under standard culture conditions, in the absence of metabolic stress. HT-29 M6 is a subpopulation of the HT-29 cell line, isolated due to its ability to grow in medium supplemented with 10^{-6} M methotrexate (MTX) (Lesuffleur et al., 1990). After confluence, HT-29 M6 cells develop a polarized phenotype, with a moderately well defined apical brush border and a cytoplasm essentially full of mucus droplets (Lesuffleur et al., 1990, 1991). By stepwise selection using higher concentrations of MTX (10^{-3} M), an HT-29 cell population with an absorptive phenotype (i.e. it does not secrete mucus and shows dome formation) has been isolated (HT-29 M3) (Lesuffleur et al., 1991). The process of differentiation has been studied extensively in HT-29-derived cell lines; the differentiated cells exhibit an increased expression of brush border associated proteins such as dipeptidylpeptidase IV (DPP-IV), carcinoembryonic antigen (CEA) or villin (Lesuffleur et al., 1990, 1991).

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acetate (TPA) stimulates proliferation or differentiation, depending on the cell line studied (Rover et al., 1979; Cohen et al., 1977; Gescher, 1985; Yuspa et al., 1982). Even in cells derived from a single epithelium the effects of TPA can be paradoxical; for instance, a subpopulation of mouse epidermal cells responds with cellular differentiation, whereas another subpopulation is stimulated to proliferate (Yuspa et al., 1982). TPA promotes its action by binding to a specific cellular receptor, protein kinase C (PK-C); TPA, binding directly, activates this enzyme (Blumberg, 1988; Jaken, 1990; Kikkawa et al., 1989). Accordingly, PK-C-mediated signal transduction is thought to be directly related to cellular growth arrest or cell proliferation in different cell types. In order to characterize the role of PK-C in intestinal epithelial cells, we have investigated the effects of TPA on the differentiation properties of the well established model of HT-29 M6 cells.

**MATERIALS AND METHODS**

**Materials**

TPA was purchased from Sigma Chemical Co. (St. Louis, MO). PK-C inhibitor GF 109203X, a gift from Dr Jorge Kirilovsky (Glaxo, Paris, France), was provided by Dr Jorge Moscat (CSIC, Madrid, Spain); it will be referred as GF. [γ-32P]ATP was from New England Nuclear. Antibodies specific for the different isoforms of PK-C were obtained from Gibco (Gaithersburg, MD). Mouse monoclonal antibodies detecting DPP-IV and villin were kindly supplied by Drs Ramón Vilella (Hospital Clínico, Barcelona) and Daniel Louvard (Institut Pasteur, Paris, France), respectively. Glycyl-L-proline-p-nitroanilide was from Bachem (Bubendorf, Switzerland). All other chemicals used were commercial products of the highest grade available.

**Cell culture**

The HT-29 cell line, established by J. Fogh (Fogh and Trempe, 1975), was supplied by Dr Alain Zweibaum (INSERM, Villejuif, France). HT-29 M6 and HT-29 M3 cells (Lesuffleur et al., 1990, 1991) were also provided by Dr Zweibaum. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco) in a humidified atmosphere of 5% CO₂, 95% air. Cells were seeded at approximately 2×10⁶ cells/cm², so that confluence was reached at days 6-7. Culture media was changed every other day to avoid nutrient depletion.

**Measurement of transepithelial resistance**

Cells were cultured onto polycarbonate filters (Transwells, Costar, Cambridge, MA) at the conditions mentioned above until 5-7 days after reaching confluence. Resistance of the monolayers was determined with a Millicel ERS Voltmeter (Millipore, Bedford, MA). After subtracting the background resistance from a filter with medium alone, values were expressed as the percentage of resistance at time 0. Typical absolute resistance values were 250-300 Ω/cm².

**Western blot analysis**

Immunoblot analyses were carried out on cell homogenates (made in 50 mM Tris-HCl, pH 7.6; 1% SDS) as previously described (Real et al., 1991) with minor modifications. Proteins were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose and incubated for 1 h with the different antibodies. Mucins were analyzed with a rabbit polyclonal antiserum raised in our laboratory by immunization with a CsCl-purified fraction of mucins secreted by HT-29 M6 cells. Detection of DPP-IV and villin was performed using the monoclonal antibodies mentioned above. PK-C isoforms were analyzed with rabbit polyclonal antiserum raised against isoform-specific peptides (α-β-γ, 8, ε or ζ). After washing with PBS, membranes were incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (Dakopatts, Copenhagen, Denmark), and reacting antigens were visualized using diaminobenzidine and H₂O₂.

**Indirect immunofluorescence**

Indirect immunofluorescence was performed on cryostat sections of cell layer rolls as reported (Lesuffleur et al., 1990). A rhodamine-conjugated goat anti-rabbit IgG (Vector, Burlington, CA) was used as secondary antibody. For immunostaining with anti-DPP-IV monoclonal antibody, an incubation with a rabbit anti-mouse Ig (Vector) was performed before adding the secondary antibody.

**DPP-IV activity**

Total cell homogenates were prepared by lysing cells in 25 mM Tris-HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl, 0.4% Nonidet P-40, at 4°C for 1 h with gentle rocking. Lysates were cleared by centrifugation at 13,000 r.p.m. for 30 min at 4°C. DPP-IV (EC 3.4.14.5) activity was measured as described (Kreisel et al., 1982), using glycyl-L-proline-p-nitroanilide as substrate. Cell number was estimated by direct counting of an equivalent sample.

**PK-C activity**

Cells were solubilized for 1 h at 4°C in 25 mM Tris-HCl, pH 7.6, 1 mM EGTA, 10 mM NaCl, 1% Triton X-100, containing 20 µg/ml leupeptin. After clearing the extracts by centrifugation, PK-C was partially purified by DEAE-cellulose chromatography (Hiraki et al., 1989). PK-C activity was assayed using either lysine-rich histone or myelin basic protein (MBP) as substrates (0.6 mg/ml in both cases) (Hiraki et al., 1989). Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay, Richmond, CA) with bovine serum albumin (BSA) as standard.

**RESULTS**

**TPA prevents and reverts the differentiation of HT-29-derived cells**

The influence of TPA on the process of differentiation of HT-29 M6 to a mucus-secreting phenotype was investigated. Ten days after confluence, HT-29 M6 and HT-29 cultures showed distinct morphologies under the phase-contrast microscope (Fig. 1A). When TPA was added to HT-29 M6 cells at the moment of confluence, their late post-confluent appearance was similar to that of HT-29 cells (Fig. 1A). The differentiated phenotype of the cells was determined by immunofluorescence, using antibodies against villin, DPP-IV and mucin. In the unselected HT-29 cell population, occasional cells expressed villin, DPP-IV or mucins; in contrast, a high proportion of cells in HT-29 M6 cultures reacted with antibodies detecting these three proteins (Fig. 1B). Villin and DPP-IV displayed an apical-cell staining pattern, whereas mucins were detected in the cytoplasm of HT-29 M6 cells as well. Similar distributions have been described in previous studies (Lesuffleur et al., 1990). As it can be observed in Fig. 1B, the addition of...
Fig. 1. TPA prevents the expression of differentiation markers during the course of HT-29 M6 culture. Control HT-29 or HT-29 M6 cells were cultured to confluence and maintained in complete medium for another 15 days with regular changes of medium. In a parallel experiment, TPA (200 nM) was added for 48 hours to a confluent HT-29 M6 culture. At day 15, phase-contrast pictures were taken (A) and cell-layer rolls prepared as described. Indirect immunofluorescence was performed using antibodies detecting villin, DPP-IV and mucins (B). This experiment was repeated twice with similar results.
TPA to HT-29 M6 cells at the time of confluence resulted in a lack of expression of villin, DPP-IV or mucin ten days later.

A more careful kinetic study of the changes in DPP-IV activity was performed. The activity of this apical enzyme correlates with its accumulation and has been widely used as a marker of differentiation of HT-29 cultures and other colon cancer cells (Lesuffleur et al., 1990, 1991; Chantret et al., 1988). DPP-IV activity of HT-29 cells was low and did not change after confluence (Fig. 2A). As reported (Lesuffleur et al., 1990), the DPP-IV activity at confluence was higher in HT-29 M6 cells than in HT-29. Contrarily to HT-29 cells, DPP-IV activity started to increase 2-3 days after confluence in HT-29 M6; by day 18 DPP-IV levels were 4-5 fold higher than at confluence (Fig. 2A). TPA completely prevented this increase in DPP-IV activity (Fig. 2A).

These effects were observed when TPA was supplemented to the culture medium for 48 hours, starting the day of confluence. Further additions of TPA to the cells did not produce a stronger effect. Although the medium was changed every other day, it has been reported that TPA binds strongly and irreversibly to the cells (Blumberg, 1988). To investigate if the TPA effect required a persistent activation of PK-C, the PK-C inhibitor GF was used. This compound, developed by Kirilovsky and co-workers, blocks PK-C activity without interfering with TPA binding to this enzyme (Toullec et al., 1991). GF has been shown to be very selective for PK-C and has been used to inhibit PK-C mediated processes in platelets and Swiss 3T3 cells (Toullec et al., 1991). In the experiment described in Fig. 2B, GF was added to the cells either at the same time, or seven days later than, TPA; DPP-IV levels were determined after seven additional days. When GF was added at the same time as TPA, the effects of this tumor promoter were completely blocked: the kinetics of DPP-IV accumulation were identical in control cells and in cells supplemented, at the same time, with TPA and GF (Fig. 2B). Moreover, GF addition to TPA-pretreated cells caused an increase in DPP-IV activity characteristic of differentiation, at a rate similar to that of control cells (Fig. 2B). These results suggested that a continuous activation of PK-C was required for the blocking of differentiation, since the inhibition of this enzyme promoted the initiation of cellular maturation.

The ability of TPA to promote the loss of differentiation markers expressed in post-confluent HT-29 M6 cells was also studied. Morphological changes could be detected as early as 1-2 hours after TPA addition (not shown). However, 2 days were necessary to decrease the levels of villin, DPP-IV and mucins to those observed in HT-29 cells (Fig. 3A). Similar results were obtained when DPP-IV activity was measured (Fig. 3B).

**Fig. 2.** TPA blocks the differentiation-related increase in DPP-IV activity in HT-29 M6 cells. 
(A) HT-29 cells, HT-29 M6 cells and HT-29 M6 cells treated with TPA (as described in the legend of Fig. 1) were incubated in complete medium; at the indicated time points, cells were lysed and DPP-IV activity was determined. Cell number was estimated from an equivalent plate. The Figure shows the averages ± s.d. of the results of five experiments. 
(B) HT-29 M6 cells were cultured in control medium (solid lines) or in medium supplemented with TPA (400 nM; dotted lines). At the indicated time-points, cells were lysed and DPP-IV activity determined. At day 7 after confluence (arrow), the cells incubated in the two mentioned experimental conditions were supplemented with GF (5 µM; open circles) or vehicle only (filled circles) and incubated for seven additional days. The kinetics of DPP-IV activity in cells treated with TPA and GF since the day of confluence were identical to the kinetics of control cells, or cells supplemented with GF. The Figure shows the results ± range of the two experiments performed.
Reversion of differentiation induced by TPA was accompanied by the loss of monolayer tight junctions. After confluence, HT-29 M6 cells form a tight layer of polarized cells. As a consequence, when cells are grown on semi-permeable filters, an electrical resistance develops between the apical and basolateral compartments (Lesuffleur et al., 1991). This transepithelial resistance (TER) is a measure of the permeability to ions of the paracellular pathway, which depends largely on the efficiency and selectivity of the tight junction barrier (Citi, 1992). Addition of TPA resulted in a quick decrease in TER, reaching values close to baseline within 30 minutes (Fig. 4). This result is consistent with a disappearance of the tight cell contacts characteristic of differentiated HT-29 M6 and necessary for cell polarization.

Optical microscopy of semi-thin sections confirmed this hypothesis. As previously described (Dahiya et al., 1992), HT-29 M6 cells form a tight layer of elongated, mostly vertically oriented cells, with some cells occasionally placed underneath them. Cells incubated with TPA for 22 hours showed a less compact structure, with greater intracellular spaces and a more rounded shape (data not shown).

Altogether, the results presented above suggest that TPA can not only prevent, but also revert, the mucus-secreting differentiated phenotype of HT-29 M6 cells. Since another HT-29-derived cell population displays an absorptive phenotype (HT-29 M3), we also investigated the effect of TPA in these cells. As shown in Table 1, the addition of TPA to HT-29 M3 cells prevented the expression of two differentiation markers: DPP-IV and the appearance of cell domes. This morphological characteristic is exclusive to polarized cells and corresponds to their ability to actively transport electrolytes across the tight cell monolayer (Zweibaum et al., 1991). TPA addition not only prevented the appearance of domes, but also caused their collapse in mature cells (not shown), probably as a consequence of the disruption of the tight junctions of the cell monolayer described above. As occurred with HT-29 M6 cells, the PK-C inhibitor GF completely prevented the effects of TPA on HT-29 M3 cells (not shown).

**PK-C activity in HT-29 M6 cells**

The results presented above suggested that the effects of TPA on the differentiated cell populations isolated from the parental HT-29 cell line were mediated through PK-C activation. However, it has been reported that very low levels of conventional PK-C (cPK-C)s are expressed in HT-29
HT-29 M3 cells were cultured in DMEM supplemented with 10% FBS (control conditions) for 12 days post-confluence. TPA (200 nM) was added to the cells for 48 hours, starting at day 2 post-confluence. The presence of cell domes was determined by exhaustive visual inspection of three independent flasks using an inverted phase-contrast microscope at ≥200 magnification. DPP-IV activity in cell extracts was determined as described. At confluence, DPP-IV activity of HT-29 M3 was 0.25 ± 0.06, not significantly different from the value obtained at 12 days post-confluence for TPA-treated cells. Averages ± ranges of two independent experiments are shown.

Table 1. TPA blocks the differentiation of HT-29 M3 cells

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Presence of cell domes</th>
<th>DPP-IV activity (units ×10^9/10^6 cells)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>+</td>
<td>1.35 ± 0.25</td>
</tr>
<tr>
<td>TPA (200 nM)</td>
<td>–</td>
<td>0.32 ± 0.05</td>
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In this article we show that PK-C activation by TPA interferes with the differentiation process of two HT-29-derived cell lines, HT-29 M6 and HT-29 M3.

The effects of TPA on cellular differentiation were studied at various levels. First, TPA prevented the expression of differentiation markers in HT-29 M6 and M3 cells. The accumulation of DPP-IV, an enzyme associated with the apical membrane of intestinal epithelial cells, was inhibited in both cell types; mucin accumulation was also blocked in the goblet cell-like HT-29 M6. TPA also reverted the expression of the differentiation markers DPP-IV, villin and mucin in differentiated cells. The half-life of DPP-IV has been shown to be very high in differentiated HT-29 cells; no decrease in the amount of [35S]methionine-labelled DPP-IV was observed by Darmoul et al. (1992) even after 48 hours of chase with nonradioactive methionine. A significant loss of DPP-IV was observed in our experiment after two days of incubation with TPA (see Fig. 3). These results suggest that the effect of TPA on this differentiation should not just be restricted to a decreased gene expression as shown in undifferentiated HT-29 cells (Darmoul et al., 1992), but should also cause a diminished protein stability. This hypothesis is currently being studied in our laboratory.

TPA also exerted a marked effect in the functional integrity of the differentiated monolayer of HT-29 cells: a complete decrease in the TER of HT-29 M6 cultures and the disappearance of domes in HT-29 M3 cells indicate that the loss of tight junctions takes place. The loss of cell-to-cell contacts was also observed using semi-thin sections and in a separate set of experiments in which homotypic aggregation assays were performed (Fabre and García de Herreros, unpublished). Furthermore, TPA was shown to induce the downregulation of E-cadherin (Fabre and García de Herreros, unpublished), a critical molecule for the intercellular adhesion of epithelial cells (Takeichi, 1991). The observation that the functional inactivation of E-cadherin blocks the ability of SW1222 colon cancer cells to form glandular structures (Pignatelli et al., 1992) reinforces the hypothesis that the overall effects of TPA on the differentiation of HT-29-derived cells might be mediated through disrupted cellular interactions.

Interestingly, the prevention and reversion of cell differentiation by TPA were not accompanied by enhanced cel-
ular proliferation. Instead, TPA induced a slight but very reproducible decrease in the growth rate of HT-29 M6 (35% ± 8, average ± s.d. of five experiments; Fabre and García de Herreros, unpublished). Similar effects in this parameter have been reported by Weinstein and collaborators in HT-29 cells that overexpressed PK-C β (Choi et al., 1990).

The analysis of protein kinase C isoforms expressed in HT-29 M6 cells has revealed very low levels of εPK-C and higher levels of the nPK-Cs ε and ζ. As the latter enzyme has been reported to be insensitive to TPA (Nakanishi and Exton, 1992), PK-C E is the main candidate for the mediator of the TPA effects on HT-29 M6 cells. However, we cannot exclude the possibility that the effects of the phorbol ester are mediated through the low levels of εPK-C present in these cells, or through some of the new nPK-Cs recently described (Bacher et al., 1991; Osada et al., 1992).

A possible role for PK-C activation in colon cancer development and progression has been suggested by several authors. In an animal model, TPA has been shown to act as a tumor promoter in the gastrointestinal tract (Goettler et al., 1979). The topical application of TPA stimulates proliferation of the epithelium of the rectal mucosa (Craven et al., 1987). However, studies with primary cultures of human colonic cells revealed that TPA stimulated the proliferation of cells derived from premalignant adenomas or preneoplastic mucosa from patients with familial polyposis, but not of normal colonic mucosa (Friedman et al., 1984). Based on these findings and the results of our own experiments, we propose that the effects of the tumor promoters on crypt proliferation might not consist in accelerating the rate of proliferation of mid-crypt cells, but in blocking the differentiation and, subsequently, the migration of these cells. Therefore, tumor promoters like TPA could, at the same time, diminish the cell growth rate and enhance the absolute proliferation of the crypt, by increasing the total number of dividing cells.

Altogether, a role for PK-C activation in the development and/or progression of colon cancer is suggested. Compounds that could directly or indirectly activate PK-C are present in the bowel content, i.e. fecal diglycerides or bile acids (Friedman et al., 1989). The exposure of normal and preneoplastic colonic cells to the bowel content offers special ways of intervention by attempting to interfere with PK-C activation. These strategies require a better understanding of the effectors of PK-C activation in the normal and preneoplastic intestinal epithelium and the biochemical characterization of the PK-C isoforms expressed in this tissue. Then, it will be possible to analyze in detail whether the inhibition of these enzymes by compounds such as GF may exert a protective or therapeutic action.

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REFERENCES


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